

C¹ cont.
contig spanning this region was constructed. Fig. 3 at b: A total of 22 polymorphic CA microsatellite markers were mapped to the contig and used in haplotype analysis in TD-1 and TD-2. Fig. 3 at c: The mutant haplotypes for probands in TD-1 and -2 indicate a significant region of homozygosity in TD-2, while the proband in TD-1 has 2 different mutant haplotypes. The candidate region can be narrowed to the region of homozygosity for CA markers in proband 2. A critical crossover at D9S1690 in TD-1 (A)* also provides a centromeric boundary for the region containing the gene. Three candidate genes in this region (*ABC1*, *LPA-R* and *RGS-3*) are shown. Fig. 3 at d: Meiotic recombinations in the FHA families (A-H) refine the minimal critical region to 1.2 cM between D9S277 and D9S1866. The heterozygosity of the TD-2 proband at D9S127, which ends a continuous region of homozygosity in TD-2, further refines the region to less than 1 cM. This is the region to which *ABC1* has been mapped. Fig. 3 at e: Isolated YAC DNA and selected markers from the region were used to probe high-density BAC grid filters, selecting BACs which via STS-content mapping produced an 800 Kb contig. Four BACs containing *ABC1* were sequenced using high-throughput methods.

[Please amend the paragraph at page 28, lines 14-21, to read as follows:

C²
Multiple DNA markers were genotyped in the region of 9q31 to which linkage to TD had been described (Rust et al., Nat. Genet. 20, 96-98, 1998). Two point linkage analysis gave a maximal peak LOD score of 6.49 at D9S1832 (Table 1) with significant evidence of linkage to all markers in a ~10 cM interval. Recombination with the most proximal marker, D9S1690 was seen in II-09 in Family TD-1 (A* in Fig. 3 at d) providing a centromeric boundary for the disease gene. Multipoint linkage analysis of these data did not increase the precision of the positioning of the disease trait locus.

[Please amend the paragraph at page 28, starting at line 22, to read as follows:

C³
A physical map spanning approximately 10 cM in this region was established with the development of a YAC contig (Fig. 3 at a). In addition, 22 other polymorphic multi-allelic markers which spanned this particular region were mapped to the contig

(Fig. 3B) and a subset of these were used in construction of a

C³
cont.

11

3 168

2

C³
C³ haplotype for further analysis (Figs. 1A and 1B; Table 2). The condensed haplotype in these families is shown in Figs. 1A and 1B.

[Please amend the paragraph at page 31, lines 3-8, to read as follows:

C⁴ While the family of Dutch descent did not demonstrate any consanguinity, the proband in TD-2 was the offspring of a first-cousin consanguineous marriage (Fig. 1B). We postulated, therefore, that it was most likely that this proband would be homozygous for the mutation while the proband in the Dutch family was likely to be a compound heterozygote. The Dutch proband shows completely different mutation bearing haplotypes, supporting this hypothesis (Fig. 3 at c).

[Please amend the paragraph at page 31, lines 9-13, to read as follows:

C⁵ The TD-2 proband was homozygous for all markers tested (Fig. 1B) distal to D9S127 but was heterozygous at D9S127 and DNA markers centromeric to it (Fig. 3 at c). This suggested that the gene for TD was likely located to the genomic region telomeric of D9S127 and encompassed by the markers demonstrating homozygosity (Fig. 3 at b).

[Please amend the paragraph at page 31, starting at line 16, to read as follows:

C⁶ Based on the defect in intracellular cholesterol transport in patients with TD, we reviewed the EST database for genes in this region which might be relevant to playing a role in this process. One gene that we reviewed as a candidate was the lysophosphatidic acid (LPA) receptor (*EDG2*) which mapped near D9S1801 (Fig. 3 at c). This receptor binds LPA and stimulates phospholipase-C (PLC), and is expressed in fibroblasts. It has previously been shown that the coordinate regulation of PLC that is necessary for normal HDL3 mediated cholesterol efflux is impaired in TD (Walter et al., J. Clin. Invest. 98:2315-2323, 1996). Therefore this gene represented an excellent candidate for the TD gene. Detailed assessment of this gene, using Northern blot and RT-PCR and sequencing analysis, revealed no

C⁶
and. changes segregating with the mutant phenotype in this family, in all likelihood excluding this gene as the cause for TD. Polymorphisms were detected, however, in the RT-PCR product, indicating expression of transcripts from both alleles.

C Please amend the paragraph at page 32, lines 4-10, to read as follows:

C⁷ The second candidate gene (*RGS3*) encodes a member of a family regulating G protein signaling which could also be involved in influencing cholesterol efflux (Mendez et al., Trans. Assoc. Amer. Phys. 104:48-53, 1991). This gene mapped 0.7 cM telomeric to the LPA-receptor (Fig. 3 at c), and is expressed in fibroblasts. It was assessed by exon-specific amplification, as its genomic organization was published (Chatterjee et al., Genomics 45:429-433, 1997). No significant sequence changes were detected.

C Please amend the paragraph at page 32, lines 11-25, to read as follows:

C⁸ The *ABC1* transporter gene had previously been mapped to 9q31, but its precise physical location had not been determined (Luciani et al., Genomics 21:150-159, 1994). The *ABC1* gene is a member of the ATP binding cassette transporters which represents a super family of highly conserved proteins involved in membrane transport of diverse substrates including amino acids, peptides, vitamins and steroid hormones (Luciani et al., Genomics 21:150-159, 1994; Dean et al., Curr. Opin. Gen. Dev. 5:779-785, 1995). Primers to the 3' UTR of this gene mapped to YACs spanning D9S306 (887-B2 and 930-D3) compatible with it being a strong candidate for TD. We initiated large scale genomic sequencing of BACs spanning approximately 800 kb around marker D9S306 (BACs 269, 274, 279 and 291) (Fig. 3 at e). The *ABC1* gene was revealed encompassing 49 exons and a minimum of 75 Kb of genomic sequence. In view of the potential function of a gene in this family as a cholesterol transporter, its expression in fibroblasts and localization to the minimal genomic segment underlying TD, we formally assessed *ABC1* as a candidate.

C Please amend the paragraph at page 36, lines 12-18, to read as follows:

C⁹ As described herein, the *ABC1* gene mapped within this interval. The overlapping genetic data strongly suggested that FHA may in fact be allelic to TD. Utilization of sets of genetic data from FHA and TD provided a telomeric boundary at D9S1866 (meiotic recombinant) (Fig. 3 at d) and a centromeric marker at D9S127 based on the homozygosity data of TD-2. This refined the locus to approximately 1 Mb between D9S127 and D9S1866. The *ABC1* gene mapped within this minimal region (Fig. 3 at e).

[Please amend the paragraph at page 41, lines 15-21, to read as follows:

C¹⁰ Version 1.7 of ClustalW was used for multiple sequence alignments with BOXSHADE for graphical enhancement (www.isrec.isb-sib.ch:8080/software/BOX_form.html) with the default parameter. A *Caenorhabditis elegans* *ABC1* orthologue was identified with BLAST (version 2.08) using CAA1005.1 (see above) as a query, with the default parameter except for doing an organism filter for *C. elegans*. The selected protein sequence has accession version number AAC69223.1 with a score of 375, and an E value of 103.

[Please amend the paragraph at page 51, lines 8-15, to read as follows:

C¹¹ Transmembrane prediction programs indicate 13 transmembrane (TM) regions, the first one being between amino acids 26 and 42 (<http://psort.nibb.ac.jp:8800/psort/helpwww2.html#ealom>). The tentative number of TM regions for the threshold 0.5 is 13. (INTEGRAL Likelihood = -7.75 Transmembrane 26-42). The other 12 TM range in value between -0.64 and -12 (full results below). It is therefore very likely that the newly-discovered 60 amino acids contain a TM domain, and that the amino end of *ABC1* may be on the opposite side of the membrane than originally thought.

[Please amend the paragraph at page 59, lines 10-25, to read as follows:

C¹² Human and rodent *ABC1* protein can be used as an antigen to raise antibodies, including monoclonal antibodies. Such antibodies will be useful for a

C¹²
correl.

wide variety of purposes, including but not limited to functional studies and the development of drug screening assays and diagnostics. Monitoring the influence of agents (e.g., drugs, compounds) on the expression or biological activity of ABC1 can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase ABC1 gene expression, protein levels, or biological activity can be monitored in clinical trials of subjects exhibiting altered ABC1 gene expression, protein levels, or biological activity. Alternatively, the effectiveness of an agent determined by a screening assay to modulate ABC1 gene expression, protein levels, or biological activity can be monitored in clinical trials of subjects exhibiting decreased altered gene expression, protein levels, or biological activity. In such clinical trials, the expression or activity of ABC1 and, preferably, other genes that have been implicated in, for example, cardiovascular disease can be used to ascertain the effectiveness of a particular drug.

Please amend the paragraph at page 64 to read as follows:

C¹³

ABC1 protein (or a polypeptide fragment thereof or an epitope-tagged form or fragment thereof) is harvested from a suitable source (e.g., from a prokaryotic expression system, eukaryotic cells, a cell-free system, or by immunoprecipitation from ABC1-expressing cells). The ABC1 polypeptide is then bound to a suitable support (e.g., nitrocellulose or an antibody or a metal agarose column in the case of, for example, a his-tagged form of ABC1). Binding to the support is preferably done under conditions that allow proteins associated with ABC1 polypeptide to remain associated with it. Such conditions may include use of buffers that minimize interference with protein-protein interactions. The binding step can be done in the presence and absence of compounds being tested for their ability to interfere with interactions between ABC1 and other molecules. If desired, other proteins (e.g., a cell lysate) are added, and allowed time to associate with the ABC polypeptide. The immobilized ABC1 polypeptide is then washed to remove proteins or other cell constituents that may be non-specifically associated with it the polypeptide or the support. The immobilized ABC1 polypeptide is then dissociated from its support, and so that proteins bound to it are released (for example, by heating), or, alternatively, associated proteins are released from ABC1 without releasing the

C¹³ *incl.* ABC1 polypeptide from the support. The released proteins and other cell constituents can be analyzed, for example, by SDS-PAGE gel electrophoresis, Western blotting and detection with specific antibodies, phosphoamino acid analysis, protease digestion, protein sequencing, or isoelectric focusing. Normal and mutant forms of ABC1 can be employed in these assays to gain additional information about which part of ABC1 a given factor is binding to. In addition, when incompletely purified polypeptide is employed, comparison of the normal and mutant forms of the protein can be used to help distinguish true binding proteins.

Please amend the paragraph beginning on page 65, line 16, to read as follows:

C¹⁴ Another assay includes a Fluorescent Resonance Energy Transfer (FRET) assay. This assay can be performed as follows.

Please amend the paragraph at page 74, lines 1-14, to read as follows:

C¹⁵ This chicken low HDL locus (Y) is Z-linked, or sex-linked. (In birds, females are ZW and males are ZZ). Genetic mapping placed the Y locus on the long arm of the Z chromosome (Bitgood, 1985), proximal to the ID locus (Bitgood, 1988). Examination of current public mapping data for the chicken genome mapping project, ChickMap (maintained by the Roslin Institute; www.ri.bbsrc.ac.uk/chickmap/ChickMapHomePage.html) showed that a region of synteny with human chromosome 9 lies on the long arm of the chicken Z chromosome (Zq) proximal to the ID locus. Evidence for this region of synteny is the location of the chicken aldolase B locus (ALDOB) within this region. The human ALDOB locus maps to chromosome 9q22.3 (The Genome Database, www.gdb.org/), not far from the location of human ABC1. This comparison of maps showed that the chicken Zq region near chicken ALDOB and the human 9q region near human ALDOB represent a region of synteny between human and chicken.